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IL-6 prevents CXCL8-induced stimulation of EpCAM expression in ovarian cancer cells

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Abstract. Epithelial cell adhesion molecule (EpCAM), which is expressed in the majority of epithelial tissues, exhibits tumor growth promoting abilities and is overexpressed in human epithelial ovarian cancer. Therefore, EpCAM is considered to be a promising target for specific immune-based therapies. The present study evaluated the role of IL-6 and IL-8 in the expression of EpCAM in the A2780 human ovarian cancer cell line. Furthermore, the cellular localization of the EpCAM protein in A2780 cells was determined and the effect of EpCAM inhibition on the proliferation of the A2780 cells was investigated. An MTT assay demonstrated that blocking EpCAM with anti-EPCAM antibodies had no effect on cellular metabolic activity (proliferation). Gene expression analysis revealed that IL-8 increased EpCAM expression, whereas IL-6 and the combination of IL-6/IL-8 had no effect on EpCAM expression. Immunofluorescence analysis confirmed that EpCAM is expressed on A2780 cell membranes. The present results demonstrated that IL-8 increased EpCAM expression at the mRNA level in ovarian cancer cells and suggested a potential role of IL-6 as an inhibitor of IL-8-stimulated EpCAM expression.

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Abbreviations: AKT, RAC-α serine/threonine-protein kinase; EpCAM, epithelial cell adhesion molecule; ERK, extracellular signal-regulated kinase; IL-6, interleukin 6; IL-8/CXCL8, interleukin 8; JAK2, Janus kinase 2; MEK, mitogen-activated protein kinase kinase; PI3K, phosphatidyl-inositol-3-kinase; SHP-2, src-homology 2 domain-containing phosphatase 2; STAT3, signal transducer and activator of transcription 3

Key words: ovarian cancer, IL-6, CXCL8/IL-8 stimulation, EpCAM expression

Introduction

Epithelial cell adhesion molecule (EpCAM) is expressed by a majority of epithelial tissues and is involved in cell signaling, proliferation, differentiation and migration. EpCAM is deregulated in epithelial malignancies and is abundantly expressed in human carcinomas of different origins (1,2). In addition to its role in cell adhesion, EpCAM acts as signaling molecule with tumor growth promoting functions. EpCAM is a part of the molecular network of oncogenic receptors and is considered to be a promising target for anti-cancer therapy (2). In addition, as a marker of aggressive ovarian cancer and an important suppressor of anti-tumor immunity, EpCAM represents an attractive target for specific immune-based therapies (1). In human epithelial ovarian cancer, EpCAM is overexpressed consistently across all histological subtypes (3,4). High-throughput genomic analysis of genetic fingerprints of primary and metastatic ovarian carcinomas demonstrates that EpCAM is one of the top differentially expressed genes in all tested epithelial ovarian cancer types (5,6). Treating breast cancer cell lines with EpCAM small interfering RNA resulted in a decrease in the rates of cell proliferation, migration and invasion; thus, these data provide compelling evidence for a direct onco- and metastatogenic role of the EpCAM protein in breast cancer (7).

Ovarian cancer progression and chemotherapy resistance depends to a great extent on the cancer microenvironment. Ovarian cancer cells have the ability to alter the composition of the microenvironment to affect host cells (8). The immune system serves an important role in ovarian cancer progression and may also be involved in the development of drug resistance via cytokine and chemokine signaling pathways (9-11).

The key cytokines in this process appear to be interleukin 6 (IL-6) and interleukin 8 (CXCL8/IL-8). Increased levels of these cytokines have been identified in ascites fluid from patients with ovarian cancer (8,12). Bonneau *et al* (13) demonstrated that alterations in IL-8 expression in ovarian tumor cells are correlated with tumor chemoresistance and overall survival, and proposed the IL-8 level as a predictor of poor prognosis in patients with ovarian cancer. IL-8 also induced epithelial-mesenchymal transition in ovarian or breast cancer cells, suggesting its potential role in enhancing ovarian cancer cell metastasis (14,15). However, an increased serum IL-6 level is additionally associated with poor prognosis in patients with early and advanced stage ovarian cancer. IL-6 promotes tumor cell migration and attachment, augmenting cancer invasiveness (16). IL-8 and IL-6 are involved in the activation of numerous cellular pathways responsible for proliferation, metastasis or tumor cell survival. Consequently, the present study evaluated the impact of IL-6 and IL-8 on the expression of EpCAM.

Materials and methods

Materials. All reagents necessary for cell cultures (RPMI-1640, bovine serum, L-glutamine and penicillin-streptomycin) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) unless indicated otherwise.

Cell culture. The human A2780 ovarian cancer cell line was obtained from the European Collection of Authenticated Cell Cultures (Salisbury, UK). A2780 cells were cultured in RPMI 1640 medium, supplemented with L-glutamine, penicillin-streptomycin (10 U/ml; 100 μg/ml) and 10% fetal bovine serum, in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C.

Cell proliferation assay following inhibition by anti-EPCAM antibodies. The effect of inhibiting EpCAM on the proliferation of the A2780 cells was determined using an MTT assay. The cells were cultured at a density of $5x10^3$ cells/well in 96-well cell culture plates (Nunc™ MicroWell™; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 24 h. The cells were treated with various concentrations of anti-EPCAM antibody (1 and 10 ng/ml; Abcam, Cambridge, UK; cat. no. ab85987) and were incubated for 30 and 60 min, and 48 h at 37˚C. The viability of the treated cells was assessed by MTT assay. Tetrazolium dye (Merck KGaA) was dissolved in PBS with Ca^{2+} and Mg^{2+} (5 mg/ml; Merck KGaA) and 15 μl of this solution was added to the cell culture. The amount of formazan dye dissolved in 10% sodium dodecyl sulfate solution was determined by quantifying its absorbance at 570 nm using the FLUOstar Omega Microplate Reader (BMG Labtech GmbH, Ortenberg, Germany). The proliferation rate (PR) was calculated via the following equation: PR (%)=(absorbance of treatment probe/absorbance of control probe) x100.

EpCAM expression

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). A2780 cells were seeded into Petri dishes ($3x10^5$ cells/ml; volume, 5 ml). After 24 h, the cells were washed with PBS with Ca^{2+} and Mg^{2+} and incubated at 37˚C for 24 h in medium containing various concentrations of IL-6 and IL-8 (1, 10 and 100 ng/ml). The total RNA was extracted using a High Pure RNA Isolation kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's protocol. The extracted RNA was diluted in DNase and RNase-free water. The quality and quantity of isolated RNA was measured using a NanoDrop® spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). Total RNA (2 μg) was reverse transcribed using High-Capacity cDNA Reverse Transcriptase [per reaction: 2 μl 10x RT Buffer; 0,8 μl 25x dNTP Mix (100 nM); 2 μl 10x RT Random Primers; 1 μl MultiScribe Reverse Transcriptase; 1 μl RNase Inhibitor; 3.2 μl nuclease-free water; Thermo Fisher Scientific, Inc.] in a final volume of 20 μl, under the following temperature conditions: 25˚C for 10 min, 37˚C for 120 min and 85˚C for 5 min. Subsequently, EpCAM expression was quantified 1 μl of the resulting cDNA solution (100 ng) using EpCAM specific TaqMan® Gene Expression assay (Assay ID, Hs00901885_m1; Thermo Fisher Scientific, Inc.) under the following temperature condition: 50˚C for 2 min; 95˚C for 30 sec; 40 cycles at 95˚C for 3 sec and 60˚C for 30 sec. The relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method (17) using β-actin gene expression as a reference.

Immunofluorescence. Cells were grown in 8-well cell culture slides (Nunc™ MicroWell™) in RPMI 1640 with 10% FBS and stimulated for 24 h with various concentrations of IL-6, IL-8 and a combination of IL-6/IL-8 (1, 10 and 100 ng/ml). Following treatment, cells were fixed in 3.7% formaldehyde for 15 min at room temperature and permeabilized in 0.1% Triton X-100 for 10 min at room temperature. Following permeabilization, the cell culture slides were blocked in 3% bovine serum albumin for 15 min at room temperature, washed and incubated with mouse monoclonal anti-EpCAM antibody (Abcam; cat. no. ab85987) at 10 μg/ml (1:100 dilution) overnight at 4˚C. Subsequently, the cells were incubated with secondary antibody (donkey anti-mouse immunoglobulin G; Alexa Fluor 488; Abcam; cat. no. ab150105; 1:1,000 dilution) for 1.5 h at room temperature. Fluorescence labeling was observed under a fluorescent microscope (BX51; Olympus Corporation, Tokyo, Japan; magnification, x400).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Multiple comparisons were performed using one-way analysis of variance followed by Tukey's post hoc test. Data are presented as the mean ± standard deviation of three replicates. All statistical tests were two-sided and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Blocking EpCAM with anti-EpCAM antibody does not affect cellular proliferation. EpCAM molecules expressed by the A2780 cell line were blocked by the addition of anti-EpCAM antibody and cell proliferation, expressed as their metabolic activity, was measured by MTT assay. However, no difference was observed between the control and treated cell proliferation rates after 30 min, 60 min and 48 h of incubation with

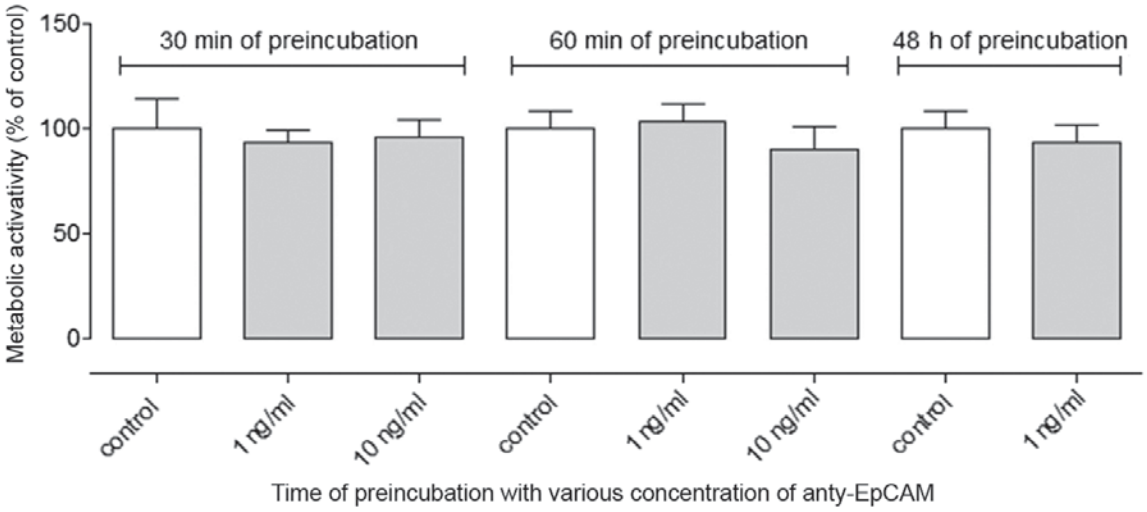

Figure 1. Cell viability evaluated by MTT assay following blocking of EpCAM by specific monoclonal anti-EpCAM antibodies. EpCAM, epithelial cell adhesion molecule.

anti-EpCAM antibody, regardless of the antibody concentration (1 or 10 ng/ml; Fig. 1).

IL-8 has a positive effect on EpCAM gene expression. RT-qPCR analysis demonstrated that the relative EpCAM expression level increased significantly in the cells stimulated with IL-8 compared with the control cells (P<0.01; Fig. 2). On the contrary, in cells treated with IL-6 (10 and 100 ng/ml) EpCAM expression did not increase significantly. Moreover, in cells incubated with the IL-6/IL-8 combination, no significant differences in expression were observed (Fig. 2).

To assess the cellular localization of the EpCAM in ovarian cancer cells, immunofluorescence analysis was performed in the A2780 ovarian cancer cell line. As hypothesized, EpCAM expression was detected at the cell membrane. However, no marked differences in fluorescence were observed between cells treated with IL-6, IL-8 or a combination of IL-6/IL-8 (Fig. 3).

Discussion

EpCAM overexpression in ovarian carcinoma, particularly in recurrent, highly metastatic, and chemotherapy-resistant ovarian cancer (18), suggests the importance of EpCAM expression for cancer growth and metastasis. Crucial evidence for a tumor promoting role of EpCAM overexpression was provided by Munz *et al* (19,20), who demonstrated upregulation of MYC proto-oncogene, bHLH transcription factor and the tumor-promoting protein epidermal-type fatty acid-binding protein by EpCAM expression. Indeed, knockdown of EpCAM resulted in suppressed proliferation and enhanced chemo- and radiosensitivity (21,22).

Positive expression of EpCAM is associated with human epithelial ovarian tumor stage and differentiation, and lymph node metastasis (23). Increased expression of EpCAM also contributes to increased viability of cancer cells *in vitro* and resistance to platinium-based chemotherapy in patients with ovarian cancer. Finally, increased expression of EpCAM is associated with a poor prognosis in patients with ovarian cancer (24). Therefore, EpCAM has begun to be a promising therapeutic target in a number of antibody-based clinical trials. In the 2009, the European Medicines Agency approved the use of an anti-EpCAM antibody, catumaxomab, for the intraperitoneal treatment of malignant ascites; however, in June 2017 the European Commission withdrew the marketing authorization for catumaxomab in the European Union (25,26).

In the present study, no effect of EpCAM inhibition on the proliferation of the A2780 cell line was observed, despite the presence of EpCAM molecules on the cell membrane. This discrepancy may have been caused by differences in the cells used, antibodies and/or time of treatment. This discrepancy may also be associated with differences in the tumor microenvironment *in vivo* and *in vitro*. Zheng *et al* (27) did not observe any effect of anti-EpCAM antibodies on the proliferation and induction of apoptosis in K562 and HL60 cells *in vitro*, even though the same antibodies inhibited the growth of EpCAM-overexpressing solid tumors and subcutaneously transplanted A549 tumors *in vivo*.

The tumor microenvironment may have a marked influence on the viability, proliferation and metastasis of ovarian cancer cells (16). The lack of expression of IL-6 and IL-8 protein, coupled with the expression of IL-6 and IL-8 receptor proteins in A2780 cells, suggests a paracrine mechanism of IL-6 and IL-8 responsivity (28,29). Therefore, it was speculated that IL-6 and IL-8, as a part of the tumor microenvironment, may influence EpCAM expression. Indeed, in the present study, IL8 was demonstrated to increase the EpCAM expression at the mRNA level, whereas treatment with IL-6 did not. Notably, co-treatment with IL-6 and IL-8 did not stimulate EpCAM expression. It may be hypothesized that src-homology 2 domain-containing phosphatase 2 (SHP-2) protein, a putative negative modulator of IL-6 signaling, may also modulate IL-8 signaling under conditions of co-stimulation. SHP-2 serves an important role in the control of proliferation, differentiation and survival of different cells (30,31). SHP-2 counteracts IL6-induced gene expression and the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway (32). Fischer *et al* (33) reported

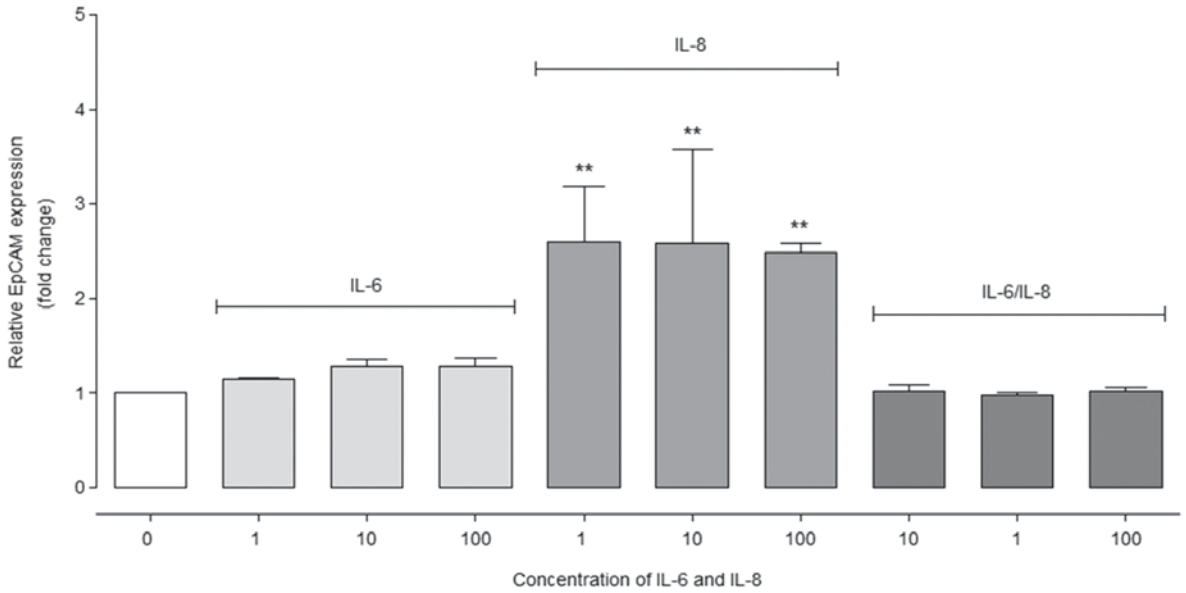

Figure 2. EpCAM mRNA expression in cells treated with IL-6, IL-8 or a combination of IL-6/IL-8. The expression level of β-actin was used as a reference gene for normalization. The data are presented as the fold change compared with the control untreated samples (designated as 0) and the data are presented as the mean ± standard deviation (n=3). **P<0.01 vs. control. IL, interleukin; EpCAM, epithelial cell adhesion molecule.

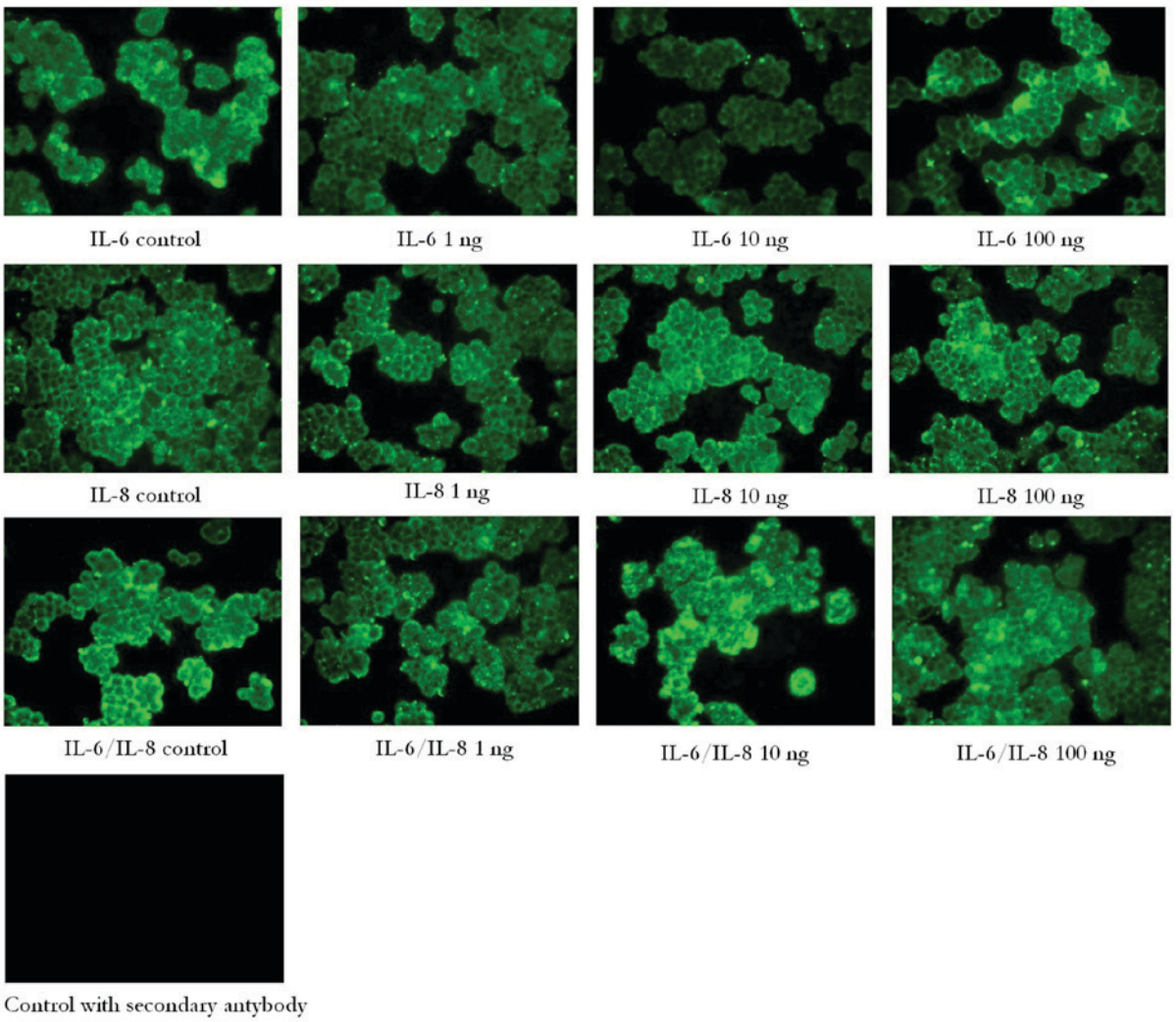

Figure 3. Immunofluorescence staining of epithelial cell adhesion molecule in the A2780 ovarian cancer cell line after 24 h of incubation with various concentrations of IL-6, IL-8 and a combination of IL-6/IL-8. Representative images of cells treated with 0, 1, 10 and 100 ng/ml of IL-6, IL-8 and IL-6/IL-8 are presented (magnification, x400). IL, interleukin.

that impaired function of SHP-2 may lead to enhanced IL-6 signaling. Another protein that regulates IL-6 signaling pathway is suppressor of cytokine signaling 3 (SOCS3). SHP-2 and SOCS3 counteract each other during IL-6-dependent gene activation (32). Mammic and Ghorpade (34) demonstrated that the SHP-2 protein is involved, directly or indirectly, in the modulation of extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) leading to CXCL8 production. In addition, SHP-2 was demonstrated to be involved in the regulation of the expression and release of IL-8 (30). It is therefore possible that SHP-2 may be involved in the regulation of gene expression associated with the signaling pathways of IL6 and IL-8.

Stimulation of the IL-6 receptor leads to activation of the JAK2/STAT3 signaling pathway, which stimulates downstream pathways involving ERK or phosphatidyl-inositol-3-kinase (PI3K)/RAC-α serine/threonine-protein kinase (AKT) kinases (35). Notably, IL-8/C-X-C chemokine receptor type 1/2 (CXCR1/2) signaling promotes the activation of similar primary effectors, e.g. PI3K/AKT, AKT, JAK2 and ERK (36). Although the two interleukins activate receptors that share certain signaling pathways, the primary targets of activation appear to differ. IL-6 receptor signaling primarily stimulates the STAT3 transcription factor pathway, whereas IL-8/CXCR1/2 signaling primarily activates PI3K or phospholipase C, promoting the activation of Akt and protein kinase C. Since EpCAM expression has been demonstrated to be stimulated by the RAF/mitogen-activated protein kinase kinase (MEK)/ERK1/2 signaling pathway (4), which is not a principal pathway activated by IL-6 or IL-8, stimulation of EpCAM expression by these interleukins may depend on the fine-tuning of secondary signaling pathways. A putative candidate appears to be an SHP-2 protein that is involved in the modulation of the RAF/MEK/ERK1/2 signaling pathway stimulated by IL-6, but not IL-8 or epidermal growth factor receptor (37). The importance of the involvement of SHP-2 in cytokine signaling remains unclear. However, certain evidence suggests that the protein negatively modulates leukemia inhibitory factor-mediated signaling (37) and is a negative effector in the cytotoxic activity of interferons, likely due to downregulation of JAK/STAT activity (38). Thus, also assuming negative modulation of IL-6 signaling, SHP-2 protein activated by IL-6 is also likely to attenuate the RAF/MEK/ERK1/2 signaling pathway activated by IL-8. However, further investigation is necessary to confirm this hypothesis.

In conclusion, the present study demonstrated that IL-8, but not IL-6, acts as an upregulator of EpCAM expression in ovarian cancer cells. It is noteworthy that co-stimulation with IL-8 and IL-6 abrogates IL-8-mediated EpCAM upregulation. The mechanism of this crosstalk remains unknown.

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Availability of data and materials

The analyzed datasets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

LKS conceived and designed the experiments. LKS, SP, KS and MC performed the experiments. LKS, BD, MK, BJJ, MMK, SP, MZD and DFT analyzed the data. LKS and SP contributed reagents/materials/analysis tools. LKS, SP, BD, MC, MMK, DFT and MZD wrote the manuscript. BJJ, BD and MK critically reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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